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DEVELOPMENT OF LYMPHOCYTE POPULATIONS IN THE HUMAN FOETAL THYMUS AND SPLEEN

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SUMMARY

T and B lymphocytes in the human foetal thymus and spleen were studied to determine the distribution and degree of development which takes place before exposure to environmental antigens occurs. Tests applied were spontaneous and complement-dependent rosette formation and immunofluorescence to detect surface immunoglobulins.

Most thymus lymphocytes were spontaneous rosette-forming cells: the percentage of these cells in the spleen was lower. Complement receptor lymphocytes (CRL) were found in the spleen but not the thymus, suggesting that these tissues contain lymphocytes of different origin. Lymphocytes with surface immunoglobulin (SIg lymphocytes) were more numerous in the spleen than the thymus. Analysis of class-specific heavy chain and light chain determinants suggests that some foetal B cells carry heavy chains of more than one class. A possible model for foetal B-cell development and its relationship to antigen drive is discussed.

INTRODUCTION

At and after 20 weeks of gestation the human foetus can respond to congenital infection with the production of plasma cells and antibody (Silverstein & Lukes, 1962). It is therefore likely that the lack of plasma cells in the normal foetus results from a lack of antigen stimulus rather than immaturity of lymphoid cells.

The process by which lymphocytes appear in the human foetal blood at 8 weeks, in the thymus by 9 weeks and in the spleen by 15 weeks (Playfair, Wolfendale & Kay, 1963) is presumably independent of antigen stimulus. Previous reports of reaction to PHA (Kay, Doe & Hockley, 1970), MLC responses (Ceppellini *et al.*, 1970) and cells with surface immunoglobulin (Lawton *et al.*, 1973) suggest that T and B lymphocytes are present in the human foetus at this early age.

We have studied T and B rosettes and surface immunofluorescence for immunoglobulin

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in foetal thymus and spleen lymphocyte preparations. These characteristics may indicate the stage to which human lymphoid cells differentiate in the absence of antigen stimulus. They also throw light on the distribution and possible source of lymphocyte sub-populations in the developing human foetus.

MATERIALS AND METHODS

Lymphocytes

Thymus and spleen were dissected from human foetuses obtained at hysterotomy and were kept in medium at 4° for up to 12 hr. Foetal age was calculated from the crown–rump length. Cell suspensions were prepared by teasing tissues in medium followed by gentle pipetting to break up cell clumps. The suspension was allowed to stand for 2 min while debris settled; the supernatant was then layered on to a Ficoll–Triosil gradient (Harris & Ukaejiofo, 1970) and centrifuged at 400 *g* for 20 min. Cells at the interface were recovered and washed three times; they were then adjusted to 1×10^7 cells per ml. More than 90% of prepared cells excluded Trypan Blue.

Medium

Tissue culture medium 199 (Wellcome Reagents) buffered with HEPES and NaOH was used throughout. For immunofluorescent procedures sodium azide was added to a final concentration of 0·1%.

Antisera

Rabbit anti-human IgG, IgM and IgA sera were purchased from Nordic and rabbit anti-human kappa and lambda from Berhingwerke. In some additional tests Wellcome sheep antihuman immunoglobulin sera (numbers IP 15, IP 16 and IP 17) were used. Antiserum to $F(ab)_2$ fragments of human IgG was raised in rabbits: it was fractionated on Sephadex G-200 and the middle peak was pooled and concentrated. Anti-IgD serum, from the Department of Experimental Pathology, Birmingham, had been raised in sheep and absorbed with insolubilized IgD-deficient human serum.

Immunofluorescent staining

An indirect immunofluorescence technique was used to detect γ, μ, α, δ, κ, λ and Fab determinants on the surface of lymphocytes. Rabbit immunoglobulin was detected with Wellcome fluorescent anti-rabbit immunoglobulin and sheep immunoglobulin with Wellcome fluorescent anti-sheep immunoglobulin.

Direct immunofluorescence tests were performed with Wellcome fluorescent anti-human immunoglobulin.

0·05–0·1 ml of cell suspension was incubated with 0·1 ml of diluted antiserum for 30 min at 0°. The cells were washed in the cold three times between and after the final additions of antiserum. They were then suspended in a drop of medium and examined immediately on a Reichert Zetopan microscope (HB 200 light source, FITC 3 interference filter, toric condenser, 40 × objective and Wratten 12 barrier filter). Each field was counted first for the number of fluorescent cells and then, with the barrier filter removed, for the total number of lymphocytes. At least 200 cells were counted in each preparation; for thymus cells up to 1000 were counted.

The optimal concentrations of the primary and secondary antisera used in the immunofluorescence tests fell in the 'plateau region'. This was established by preliminary titration in which human adenoid lymphocyte preparations were stained with doubling dilutions of the antisera.

Purified human polyclonal IgG and IgA and myeloma IgM were coupled to a cellulose absorbent (Sigmacell Type 19, Sigma Chemical Company) activated with cyanogen bromide (McLaughlan *et al.*, 1971). These particles were then processed in the same way as lymphocytes and were examined by direct and indirect immunofluorescence. Methanol-fixed preparations of two IgG myeloma marrows were also stained with the indirect immunofluorescence technique.

Controls entailing blocking of the second stage fluorescent antiserum by unlabelled antisera, omission of antisera or substitution of normal rabbit immunoglobulin, were also done.

Spontaneous rosette-forming cells (SRFC) test

Sheep erythrocytes (Wellcome Reagents) <10 days old, were washed three times in medium and adjusted to 1% by volume. 0·1 ml of this suspension and 3×10^5 lymphocytes in 0·1 ml medium were mixed vigorously in 7-mm round-bottomed plastic tubes and centrifuged at room temperature for 7 min at 150 *g*. The tubes were then placed in an ice bath for 2 hr. The cells were resuspended by gentle rocking and viewed on a microscope (direct and phase illumination) immediately.

Complement receptor lymphocyte (CRL) test

Sheep erythrocytes were washed, adjusted to 5% and sensitized with rabbit anti-sheep red cell serum (Wellcome Reagents) at a final dilution of 1:2000 for 30 min at 37°. They were then washed and incubated with fresh human serum diluted 1:40 for 30 min at 37°. 10^7 sensitized SRBC (EAC) were mixed with 5×10^5 lymphocytes in 7-mm round-bottomed plastic tubes and centrifuged at room temperature for 5 min at 150 *g*. The tubes were incubated at 37° for 30 min and the cells were then resuspended with a vortex mixer; they were examined immediately under a microscope.

RESULTS

Adequate numbers of cells for study were obtained, after Ficoll separation, from all foetal thymuses. Insufficient numbers of cells could be recovered from the gradient from foetal spleens of less than 15 weeks gestation, so only crude suspensions were studied. Between 15 and 19 weeks sufficient lymphocytes for some, but not all, tests were obtained.

More than 90% of the cells prepared from foetal thymus were small or medium-sized lymphocytes: less than 10% were erythrocytes. Spleen cell preparations contained lymphocytes with similar morphology but also up to 80% erythrocytes. In addition up to 30% of the mononuclear cells were large with abundant granular cytoplasm, these were easily distinguished by light and UV microscopy and were excluded from the counts.

Spontaneous rosette-forming cell test

Fifty to 96% of thymus lymphocytes from thirteen foetuses formed spontaneous rosettes (Fig. 1). There is a trend towards lower values below 18 weeks of gestation. Fewer (0–41%) of spleen cells were SRFC.

Spontaneous rosette formation by foetal thymus cells was not inhibited by addition of anti-F(ab)$_2$ serum.

Complement receptor lymphocyte test

No (<0·5%) lymphocytes of the thymuses of five foetuses of 19–24 weeks gestation made rosettes with EAC, only one (0·6%) was seen in the other. Nineteen to 23% (mean 21%, s.d. 1·87%) of spleen lymphocytes from six foetuses did so. Under conditions of the test neither spleen nor thymus lymphocytes made rosettes with EA in the absence of complement.

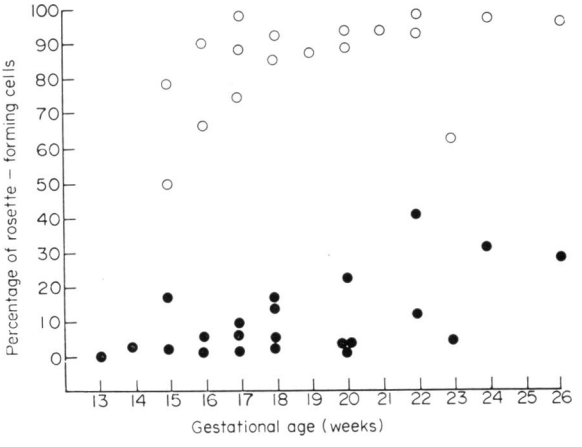

FIG. 1. The percentage of spontaneous rosette-forming cells in (○) foetal thymus and (●) spleen suspensions plotted against gestational age.

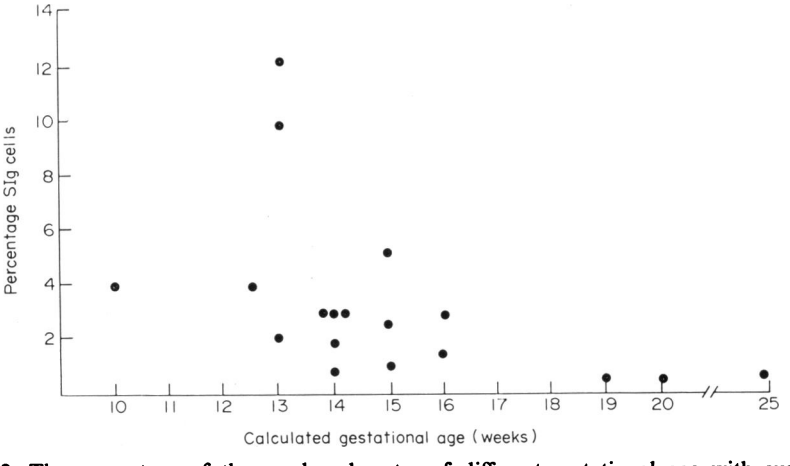

FIG. 2. The percentage of thymus lymphocytes of different gestational age with surface immunoglobulin detected with anti-F(ab)$_2$ reagent.

Immunofluorescence

Cells classified as SIg had a bright peripheral rim of fluorescence which capped in the absence of sodium azide.

Few SIg lymphocytes were found in the thymus preparations at any age. The percentage staining with the anti-F(ab)$_2$ reagent, which would detect all immunoglobulins, is plotted in Fig. 2. Positive cells were observed in all foetuses studied and there was a fall with gestational age (r = 0·71, α<0·01, Spearman rank correlation).

TABLE 1. SIg Lymphocytes in foetal thymus suspensions

Age in weeks	Percentage of lymphocytes staining for:				
	γ	μ	α	δ	F(ab)$_2$
10	n.d.	n.d.	n.d.	n.d.	4·1
12½	n.d.	n.d.	n.d.	n.d.	4
13	0	0·05	n.d.	n.d.	8
14	1·6	0·5	0·45	0·3	2·4
15	0·6	0·3	0	0	3
16	0·003	n.d.	n.d.	n.d.	2·4
19	0	0·2	0	0	0·6
20	0	0·2	0·3	0·5	0·5
25	0·2	0·4	0	0·4	0·5

Mean values are shown where more than one foetus was examined.
n.d. = not done.

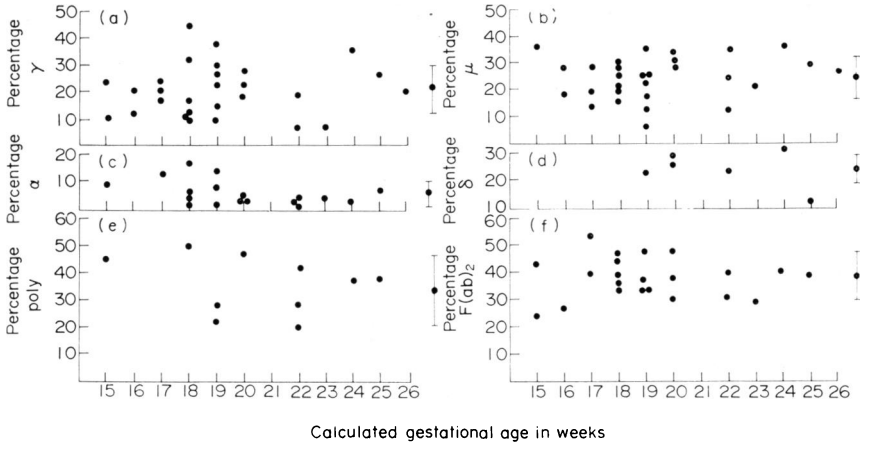

FIG. 3. The percentage of foetal spleen lymphocytes with surface immunoglobulin detected by immunofluorescence plotted against gestational age. Antisera used are: (a) anti-γ; (b) anti-μ; (c) anti-α; (d) anti-δ; (e) polyvalent anti-immunoglobulin; (f) anti-F(ab)$_2$. Means are shown ± one standard deviation at the right hand side of each graph.

The percentage staining for each of γ, μ, α and δ (the heavy chains of IgG, IgM, IgA and IgD respectively) was lower and positive lymphocytes were not always detected. Values at different ages (Table 1) do not suggest preponderance of any one class.

SIg lymphocytes were much more common in foetal spleen: results from thirty spleens

age 15–25 weeks gestation are plotted against age in Fig. 3a–f. There is no obvious relationship with age.

Sufficient cells were obtained from only six spleens for most of the tests to be performed in parallel: these were all from foetuses of 19 weeks or over.

The results (Table 2) show a similar percentage of lymphocytes stained with the polyvalent anti-immunoglobulin serum and the indirect anti-$F(ab)_2$ reagent. The combined percentages of lymphocytes staining with antisera to γ, μ, α and δ was consistently greater than this. In two instances the combined percentages of SIg lymphocytes and those which were SRFC exceeded 100%. The percentage of CRL was always less than the percentage of SIg lymphocytes.

TABLE 2. Surface immunoglobulins on foetal spleen lymphocytes detected by immunofluorescence with specific antisera

Gestational age in weeks	Percentage of lymphocytes with heavy chains				Summed percentage of $\gamma+\mu+\alpha+\delta$	Percentage stained by		CRL (%)	SRFC (%)
	γ	μ	α	δ		Polyvalent antiserum	Anti-$F(ab)_2$ reagent		
19	29·5	25	8	24	86·5	28	28	20	n.d.
20	25	26	8	27	86	n.d.	n.d.	23	n.d.
20	28·5	29	4·8	30	92·3	49	48	20	2·5
22	19	28	7	25	79	28	39	19	4
24	36	35	3	33	107	37	40	23	n.d.
25	26·9	27·9	6·4	13·7	74·9	37	37·2	n.d.	35·1

n.d. = not done.

TABLE 3. Surface light chains on human foetal spleen lymphocytes

Age in weeks	Percentage κ	Percentage λ	Percentage $F(ab)_2$
20	35	12	48
24	16	6	40
25	25·5	11·8	37·2

In each of the crude spleen suspensions from the three youngest spleens studied (13, 14 and 14 weeks) 1–2% of cells staining with the anti-$F(ab)_2$ reagent were found.

Staining with light chain class-specific antisera and the anti-$F(ab)_2$ reagent was studied in three spleens (Table 3). In each κ outnumbered λ SIg cells, and the serum of the two did not exceed the proportion staining with anti-$F(ab)_2$.

Controls

Anti-human immunoglobulin antisera. Immunoelectrophoretic analysis showed that the rabbit anti-human immunoglobulin sera formed precipitation lines with the homologous immunoglobulin only. The IgG fraction of rabbit anti-$F(ab)_2$ reacted with IgG, IgM and IgA. The anti-IgD reacted with IgD myeloma protein but not with IgG, IgM or IgA.

Bright staining of immunoglobulin-coated Sigma cell particles was seen when the corresponding anti-immunoglobulin reagents were used: other combinations produced no staining. The optimal dilutions are shown in Table 4.

The myeloma marrow preparations were stained by the anti-γ but not by the anti-μ or anti-α.

TABLE 4. Specific antisera used in immunofluorescent tests

Source	Code	Specificity	Staining of Sigma cell coated with			Final dilution
			IgG	IgM	IgA	
Wellcome	IP 16	IgG	+	−	−	1:24
Wellcome	IP 17	IgM	−	+	−	1:24
Wellcome	IP 15	IgA	−	−	+	1:16
Wellcome	MF 01	Polyvalent	+	+	+	1:4
Wellcome		Anti-sheep	−	−	−	1:20
Wellcome		Anti-rabbit	−	−	−	1:20
Nordic	Ra/Hu IgG	IgG	+	−	−	1:32
Nordic	Ra/Hu IgM	IgM	−	+	−	1:32
Nordic	Ra/Hu IgA	IgA	−	−	+	1:20

0·1 ml of each diluted class-specific rabbit anti-human immunoglobulin serum was incubated with 1 mg of glutaraldehyde insolubilized IgG for 30 min at room temperature. This mixture was then centrifuged and cooled and used in the first stage of the indirect immunofluorescence staining of foetal spleen cells. Tracing with the fluorescent antisera showed that γ staining was inhibited but the percentage of cells staining for μ and α was unchanged. δ staining of spleen cells was inhibited when anti-D serum was preincubated with an IgD myeloma serum, but not with normal serum. Five foetal spleen suspensions were stained in parallel with the Nordic (rabbit) and the Wellcome (sheep) class-specific antisera to human immunoglobulins; the percentage of positive cells corresponded to within 5%.

Anti-species fluorescent antisera. Neither the anti-sheep nor the anti-rabbit antisera stained immunoglobulin-coated Sigmacell particles. No foetal thymus cells and only 1 in 500 spleen cells stained following incubation with these antisera alone.

Anti-immunoglobulin staining of foetal spleen cells was inhibited if an incubation step with anti-rabbit blocking serum was introduced between the first and second stages of the indirect procedure.

SIg lymphocytes in adult blood. The mean values of SIg lymphocytes in normal adult blood obtained with these reagents in this laboratory are: IgG = 12·6%; IgM = 8%; IgA = 4%.

DISCUSSION

SIg lymphocytes and SRFC were found in different proportions in foetal thymus and spleen suspensions. The high percentage of SRFC in foetal thymus resembles our experience and

published reports (Jondal, Holm & Wigzell, 1972) of SRFC in adult thymus and is consistent with current interpretations of this test as a marker for T lymphocytes. Brain & Marston (1973) were unable to inhibit the SRFC formation by adult blood lymphocytes with anti-immunoglobulin sera and we were unable to do so in foetal thymus lymphocytes. This contrasts with the observation by Stites *et al.* (1972) of non-age-related inhibition of thymus SRFC by anti-γ serum and an age-related inhibition by anti-μ serum. We found a higher percentage of SRFC in foetal thymus than Stites *et al.* (1972) and Whittingham & Mackay (1973); this is probably due to differences in technique.

We saw few SIg cells in foetal thymus preparations, confirming the findings of Lawton *et al.* (1973). The greatest proportion is found early in gestation when development of the thymus vascular bed is further advanced than that of the parenchyma, and it is possible that thymus SIg cells belong to the blood rather than thymus lymphocyte compartments.

SIg cells consistently outnumbered SRFC in the foetal spleen and this difference was greatest at 20 weeks' gestation. The different characteristics of the majority of foetal thymus and spleen cells suggest that these tissues include functionally different lymphocyte populations which arise independently. The largest proportion of SRFC in foetal spleen was found at 22 weeks. This could be due to peripheralization of thymus-derived cells and is compatible with the increase in blood lymphocyte counts reported to occur at 20 weeks (Playfair *et al.*, 1963). Some B cells are also CRL (Ross *et al.*, 1973) and such cells were detected in significant numbers only in the spleen, not in the thymus. Consequently it is unlikely that large scale lymphocyte traffic takes place from the spleen to the thymus.

Lymphocyte surface immunoglobulin is thought to have receptor function and to be an accurate sample of the antibody specificity of the cell. We do not know what the specificities of most foetal lymphocytes are although the demonstration of antigen binding by a proportion of such cells confirms that these specificities exist (Dwyer & Mackay, 1970; Hayward & Soothill, 1972).

SIg cells, observed in the foetal spleen as early as 13 weeks' gestation, presumably also have antigen specificity expressed in the variable regions of the heavy and light chains. The observation of surface γ, μ, α and δ heavy chains suggests that immunoglobulins of different classes arise in man independent of antigen stimulus. This process may be analogous to that observed in the chicken bursa by Kincade & Cooper (1972) and Hudson, Thantrey & Roitt (1974). However, in contrast to Hudson *et al.*, we found no evidence of sequential maturation of different heavy chain classes.

The sum of the percentages of spleen lymphocytes staining for the different heavy chain classes was much higher than has been reported for any other non-malignant lymphoid population. The percentage of lymphocytes stained directly with the polyvalent anti-human immunoglobulin serum was similar to that stained indirectly by the anti-$F(ab)_2$ reagent. This suggests that the proportion of foetal spleen lymphocytes with surface heavy chains was not greater than that with surface light chains. The percentage of spleen lymphocytes with surface light chains (mean 37%) was far fewer than the combined percentages with surface heavy chains (mean 77·4%); this could be ascribed to simultaneous carriage of more than one heavy chain class.

From the results of Rowe *et al.* (1973b) it is likely that simultaneous carriage of μ and δ heavy chains by foetal spleen lymphocytes has contributed to our findings. The γ chains which we detected on many foetal spleen lymphocytes could have been synthesized locally since labelled amino acid incorporation into IgG by foetal tissue fragments has been found

as early as 12 weeks' gestation (Gitlin & Biasucci, 1969). Alternatively the IgG may have been acquired from the mother (Dancis *et al.*, 1961) and have bound to Fc receptors on B lymphocytes (Dickler & Kunkel, 1972). Experiments to distinguish between these possibilities are under way.

Kincade & Cooper (1971) and Hudson, Thantrey & Roitt (1974) concluded that developing B cells in the chicken bursa could carry immunoglobulins of more than one heavy chain class. Our findings suggest that the foetal spleen may be a primary lymphoid organ where B cells develop in the human foetus.

The significance of multiple heavy chain carriage on foetal B cells is unclear. It is unlikely to be due to sequential maturation of B cells from one heavy chain class to another, since the proportions of cells staining with individual antisera varied little with gestational age. One possibility is that individual spleen lymphocytes are able to synthesize surface immunoglobulins of more than one heavy chain class because repression of these heavy chain genes has not yet occurred. This suggests parallels with the development of antigen-binding cells in the human foetal thymus where evidence of genetic instability was also found (Hayward & Soothill, 1973). We suggest that virgin B cells (Bx in the notation of Sercarz & Byers, 1967), are characterized by surface immunoglobulins of multiple heavy chain classes, including δ. A proportion of these cells are also CRL.

Many of the 18% of IgD-bearing cells observed by Rowe *et al.* (1973b) in cord blood also carry IgM. It is therefore likely that unrepressed B cells persist in the circulation until birth. The much lower proportion of δ lymphocytes (Rowe *et al.*, 1973a) and low number of multiple staining cells in adult blood could be the consequence of antigen-driven maturational steps, from Bx to By (memory) and Bz (plasma) cells (Sercarz & Byers, 1967). If this is so the B cells found in adult blood, which appear to carry only one heavy chain class, would be predominantly By cells. They would start to accumulate after birth as a consequence of exposure to environmental antigens which would result in repression of genes for all but one heavy chain class.

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